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Partial *AZFc* duplications not deletions are associated with male infertility in the Yi population of Yunnan Province, China*

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Abstract: Objective: There are many reports on associations between spermatogenesis and partial *azoospermia factor c* (*AZFc*) deletions as well as duplications; however, results are conflicting, possibly due to differences in methodology and ethnic background. The purpose of this study is to investigate the association of *AZFc* polymorphisms and male infertility in the Yi ethnic population, residents within Yunnan Province, China. Methods: A total of 224 infertile patients and 153 fertile subjects were selected in the Yi ethnic population. The study was performed by sequence-tagged site plus/minus (STS+/−) analysis followed by gene dosage and gene copy definition analysis. Y haplotypes of 215 cases and 115 controls were defined by 12 binary markers using single nucleotide polymorphism on Y chromosome (Y-SNP) multiplex assays based on single base primer extension technology. Results: The distribution of Y haplotypes was not significantly different between the case and control groups. The frequencies of both gr/gr (7.6% vs. 8.5%) and b2/b3 (6.3% vs. 8.5%) deletions do not show significant differences. Similarly, single nucleotide variant (SNV) analysis shows no significant difference of gene copy definition between the cases and controls. However, the frequency of partial duplications in the infertile group (4.0%) is significantly higher than that in the control group (0.7%). Further, we found a case with sY1206 deletion which had two *CDY1* copies but removed half of *DAZ* genes. Conclusions: Our results show that male infertility is associated with partial *AZFc* duplications, but neither gr/gr nor b2/b3 deletions, suggesting that partial *AZFc* duplications rather than deletions are risk factors for male infertility in Chinese-Yi population.

Key words: *Azoospermia factor c* (*AZFc*), *AZFc* polymorphism, b2/b3, gr/gr, Infertility
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1 Introduction

The *azoospermia factor* (*AZF*) region, mapped to Yq11, is associated with spermatogenesis (Bardoni *et al*., 1991; Vog *et al*., 1996; Pryor *et al*., 1997). There are three subregions within *AZF*, referred as *AZFa*, *AZFb*, and *AZFc* (Vog *et al*., 1996). Today, testing for microdeletions of *AZF* is routine in cases of male infertility. *AZFc* is comprised of long direct and inverted repetitive sequence blocks called "amplicons", which make *AZFc* one of the most unstable

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regions in the human genome (Fig. 1a) (Kuroda-Kawaguchi *et al*., 2001; Skaletsky *et al*., 2003). The *AZFc* deletion, also known as b2/b4 deletion, leads to variable phenotypes ranging from azoospermia to oligozoospermia. It is the most commonly known genetic cause of spermatogenic failure (Ferlin *et al*., 2007; Simoni *et al*., 2008). In this region, three copies of *basic protein Y2* (*BPY2*), two copies of *chromodomain Y1* (*CDY1*), and four copies of *deleted in azoospermia* (*DAZ*) have been identified (Fig. 1a) (Navarro-Costa *et al*., 2010b). These genes are considered as candidates important for spermatogenesis. In addition to the 3.5-Mb *AZFc* deletion, several partial deletions in this region have been reported, such as gr/gr and b2/b3 deletions, both of which remove almost half of the *AZFc* gene copies, including two copies of *DAZ* and one copy of *CDY1* (Figs. 1b–1e) (Repping *et al*., 2003; 2004). However, the reports on associations between partial *AZFc* deletions and infertility have been conflicting (Kuroda-Kawaguchi *et al*., 2001; Giachini *et al*., 2008). Several studies about partial *AZFc* deletions and infertility have been performed in Chinese-Han population, but results have been conflicting and may be due to control selection, sample quantity, different ethnic backgrounds, or geographic areas (Zhang *et al*., 2006; Lin *et al*., 2007; Wu *et al*., 2007; Lu *et al*., 2009; Yang *et al*., 2010). Polymorphic expression among DAZ proteins has been reported, indicating unequal activities of different DAZ copies (Kim *et al*., 2009). Thus, analysis on deletion types of *DAZ* or other related genes is necessary to determine the relationship between partial *AZFc* polymorphisms and male infertility. In addition to the partial *AZFc* deletions, partial duplications in this region have been identified (Lin *et al*., 2005). Associations between partial duplications and male infertility have been found in the Chinese-Han population, but not in the Italian population (Lin *et al*., 2007; Giachini *et al*., 2008).

Though lots of studies have been carried out, the impact of partial *AZFc* deletions and duplications on spermatogenesis is inconclusive. In this paper, we report an association study on *AZFc* polymorphisms and male infertility in the Yi population, an ethnic minority in southwestern China. We carefully selected 224 infertile patients and 153 fertile controls. We did not find significant frequency differences between the case and control groups for partial *AZFc* deletions and subtypes analyzed by single nucleotide variant (SNV). However, the frequency of partial *AZFc* duplications in infertile patients was significantly higher than that in fertile men. Our results indicate that partial *AZFc* duplications, rather than deletions, are likely risk factors for male infertility in the Chinese-Yi population.

2 Materials and methods

2.1 Subjects

A total of 224 infertile patients and 153 control subjects were selected from the Population and Family Planning Institute in Yunnan Province, China. All the samples were from the Yi population in Yunnan Province with normal 46,XY karyotype. The men with Y-chromosome microdeletions were excluded. The infertile men had either non-obstructive azoospermia or severe oligozoospermia with a sperm count $<5\times10^6$ spermatozoa/ml, on the basis of repeated semen analysis according to the World Health Organization (WHO) criteria. The subjects with history of orchitis and active orchitis, history of unilateral or bilateral cryptorchidism and varicocele, and hypogonadotrophic hypogonadism were also excluded from the infertile group. The fertile controls were healthy men who had given birth to at least one offspring without the help of assisted reproductive technology, which had been confirmed by paternity test.

2.2 Sequence tagged site plus/minus (STS+/−) analysis of *AZFc*

Overall, six *AZFc* specific STSs (sY1191, sY1291, sY1206, sY1201, sY142, sY1161) were screened for all the samples by polymerase chain reaction (PCR) analysis (Fig. 1a). The gr/gr deletion was represented by the absence of sY1291 with the presence of the other STSs. The b2/b3 was represented by the specific absence of sY1191. The primer sequences and PCR conditions were as described previously (Fernandes *et al*., 2002; Repping *et al*., 2003). For case 3, additional STSs (sY254, sY639, sY1054, sY1125) were screened (Fig. 1a).

2.3 SNV analyses of *DAZ* and *CDY1* genes

The samples with the absence of sY1191 or sY1291 were carried out by SNV analysis using

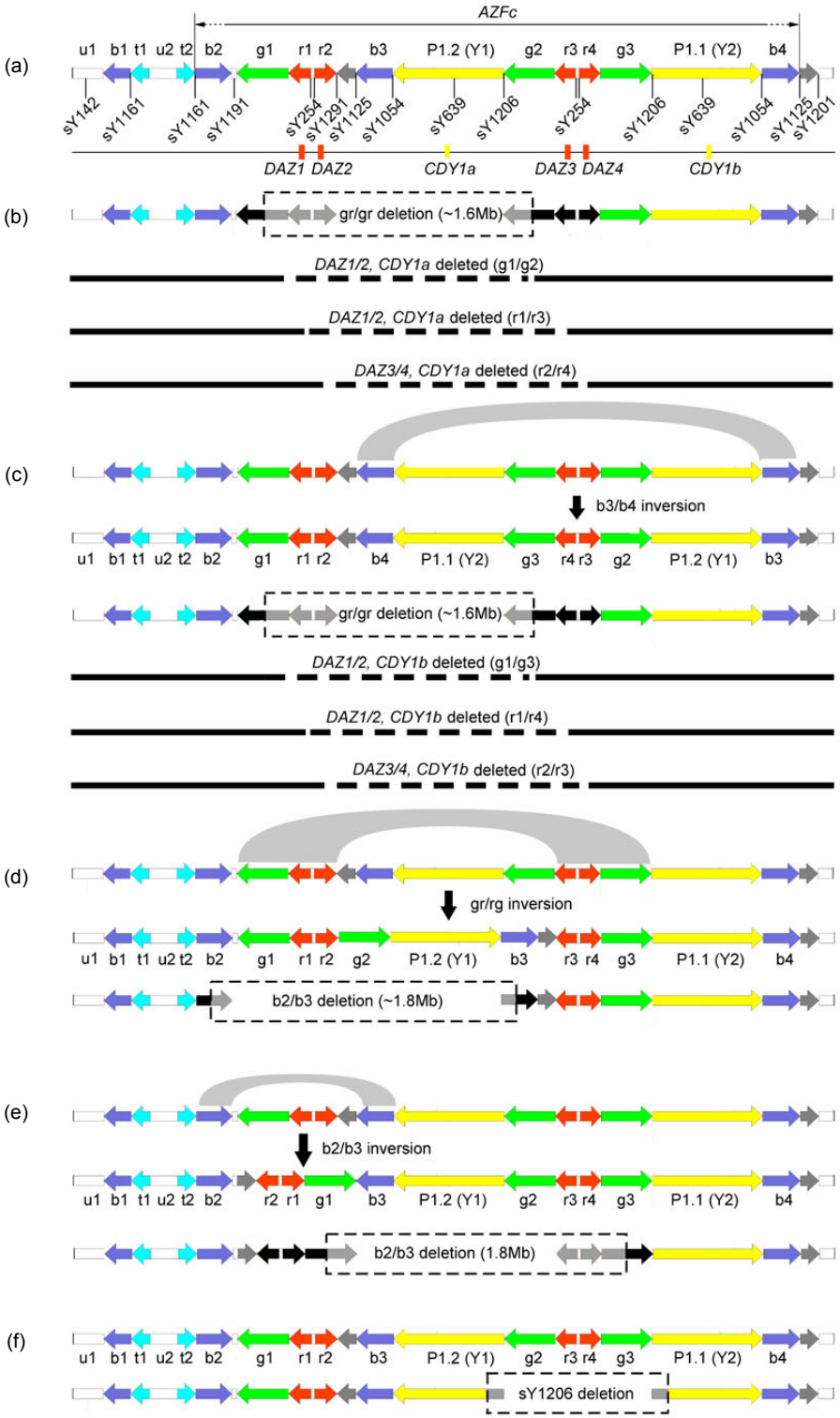

Fig. 1 Representation of *AZFc* structure and arrangement

(a) The structures of amplicons, locations of sequence-tagged sites, and transcription units of *DAZ* and *CDY1* gene families (Zhang *et al*., 2007); (b) The gr/gr deletion and sub-types (Krausz *et al*., 2009); (c) The gr/gr deletion following b3/b4 inversion and sub-types (Krausz *et al*., 2009); (d) The b2/b3 deletion following gr/rg inversion (Zhang *et al*., 2007); (e) The b2/b3 deletion following b2/b3 inversion (Zhang *et al*., 2007); (f) The sY1206 deletion

a PCR amplification-restriction digestion assay. SNV sY587 and CDY7750 were chosen for distinguishing *DAZ1/2* from *DAZ3/4*, and *CDY1a* from *CDY1b*, respectively. The methods were according to Machev *et al.* (2004).

2.4 *DAZ* and *CDY1* gene dosages

The dosage analyses of *DAZ* and *CDY1* were performed as previously described (Machev *et al*., 2004; Yang *et al*., 2010). Primer pair o1130/o1313 was used to amplify *DAZ* (214 bp) and *DAZL* (217 bp), and oMY953a/o1023 was for amplification of *CDY1* (134 bp) and *CDY2* (137 bp). o1130 and oMY953a were labeled with 5′ FAM (Invitrogen). The PCR products were analyzed using ABI PRISM 3100 Avant genetic analyzer (Applied Biosystems).

2.5 Y chromosome haplogrouping

Y chromosome haplogroups were defined by 12 binary markers: M130, M174, M9, M175, M119, M95, M122, M324, P201, M7, M134, and M117. These single nucleotide polymorphisms (SNPs) were detected by Y-SNP multiplex assays based on single base primer extension technology using SNaPshot Multiplex kit (Applied Biosystems), which was performed as previously described (van Oven *et al*., 2011). Primers M174, M9, and M175 were designed as described by van Oven *et al*. (2011). The other primer sequences were kindly provided by Dr. Bo-wen CHENG (unpublished). These 12 markers defined 12 haplogroups, following the recommendation of Shi *et al*. (2005).

2.6 Statistical analysis

The frequencies of partial *AZFc* deletions, duplications, partial deletion sub-types, and the distribution of haplotypes between the cases and controls, were compared by Chi-squared test ($P<0.05$ was regarded as statistically significant) using the statistical package SPSS 10.0 (SPSS Inc.).

3 Results

3.1 Partial *AZFc* deletions

Based on the STS+/− analysis, gr/gr and b2/b3 deletions were detected in both infertile men and fertile control subjects, but no b1/b3 deletions were found (Figs. 1a–1e and 2). We found 4 men in the azoospermia group and 13 in the severe oligozoospermia group with sY1291 absent (Table 1). Among the 17 gr/gr deletions in the infertile men, 15 were simple gr/gr deletions, 1 was gr/gr deletion with b2/b4 duplication (four *DAZ* copies and two *CDY1* copies) and 1 was coupled with multiple b2/b4 duplications (six *DAZ* copies and three *CDY1* copies) based on the *DAZ* and *CDY* copy analysis (data not shown). Thirteen fertile men were found with gr/gr deletions, all of which were simple gr/gr deletion (Table 1). The frequency of the gr/gr deletion did not show significant differences between the case and control groups.

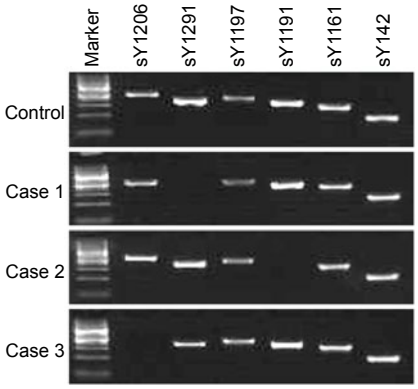

Fig. 2 Detection of the partial *AZFc* deletions with specific *AZFc* STSs
Marker: 100 bp ladder; Control: fertile man; Case 1: gr/gr deletion; Case 2: b2/b3 deletion; Case 3: sY1206 deletion

Table 1 Frequencies of partial *AZFc* deletions

Group	*n*	gr/gr deletion	b2/b3 deletion	sY1206 deletion
Fertile males	153	13 (8.5%)	13 (8.5%)	0 (0.0%)
Azoospermia	55	4 (7.3%)	2 (3.6%)	0 (0.0%)
Severe oligozoospermia	169	13 (7.7%)	12 (7.1%)	1 (0.6%)
Infertile males	224	17 (7.6%)	14 (6.3%)	1 (0.4%)

Fourteen cases (2 in azoospermia and 12 in severe oligozoospermia), and 13 controls were found with b2/b3 deletion, among which 10 and 11 were simple deletions, respectively (Table 1). The others were coupled with b2/b4 duplications. No significant difference was found in this type of deletion between the infertile and fertile groups.

In addition, we found a new deletion type on a patient bearing severe oligozoospermia. In this case,

sY1206 was negative while the other five sites were positive (Fig. 2, Table 1). To confirm the results and investigate the breakpoints, we tested other related STSs, including sY254, sY639, sY1054, and sY1125. All of these STSs were positive (data not shown), indicating that the breakpoints located between sY1206 and sY1054, namely between Y1 and Y2 (Fig. 1f) (Navarro-Costa *et al*., 2010a).

3.2 SNV analysis

We discriminated *DAZ1/2* from *DAZ3/4* and *CDY1a* from *CDY1b* by SNV analysis, which divided partial deletions into four sub-types: *DAZ1/2+CDY1a*, *DAZ1/2+CDY1b*, *DAZ3/4+CDY1a*, and *DAZ3/4+CDY1b* deletions (Figs. 1b–1e). Among the 15 simple gr/gr deletions in cases, no *DAZ3/4+CDY1a* deletion was found, while 5 samples were found for the other three sub-groups, respectively (Table 2). The 13 gr/gr deletions in the control group were sub-grouped into *DAZ1/2+CDY1a* deletions (8) and *DAZ3/4+CDY1b* deletions (5) (Table 2). Five cases with the *DAZ1/2+CDY1b* deletion were identified, in contrast to none in the controls; however, the difference was not significant (P=0.064). All b2/b3 deletions in cases were grouped into *DAZ3/4+CDY1b*, while in the control group besides this type of deletion, one *DAZ1/2+CDY1a* deletion and one *DAZ1/2+CDY1b* deletion were found (Table 2). Case 3, with sY1206 missing, was sub-grouped into *DAZ3/4+CDY1a* deletion (data not shown).

3.3 *DAZ* and *CDY1* gene dosages

Besides partial deletions, partial duplications are reported (Repping *et al*., 2003; Machev *et al*., 2004; Giachini *et al*., 2005). We analyzed the duplications by detecting the copy numbers of *DAZ* and *CDY1* (Fig. 3). The partial duplications with six copies of *DAZ* and three copies of *CDY1* were found in both infertile (9) and fertile (1) groups (Table 3), which showed significantly different frequencies (P<0.05). The frequency for this type of partial duplication is higher in the severe oligozoospermia group (4.7%) than in the azoospermia group (1.8%).

In case 3, two copies of *CDY1* were detected by dosage analysis, but only the *CDY1b* band was detected by SNV analysis, which was probably caused by gene conversion (Fig. 3). Only two copies of *DAZ* were detected, which turned out to be *DAZ1/2* (Fig. 3).

3.4 Y chromosome haplogroups

It has been suggested that different haplogroups show diverse frequencies of partial *AZFc* deletions (Lin *et al*., 2007; Zhang *et al*., 2007). To investigate the haplogrouping of the samples in this study, 215 cases and 115 controls were tested and grouped by 12 Y chromosome markers (Fig. 4). The distribution of haplotypes was not significantly different between the cases and controls (Table 4). Partial *AZFc* deletions are obviously clustered in haplogroup K-R* (* indicates that the haplogroup may not be monophyletic).

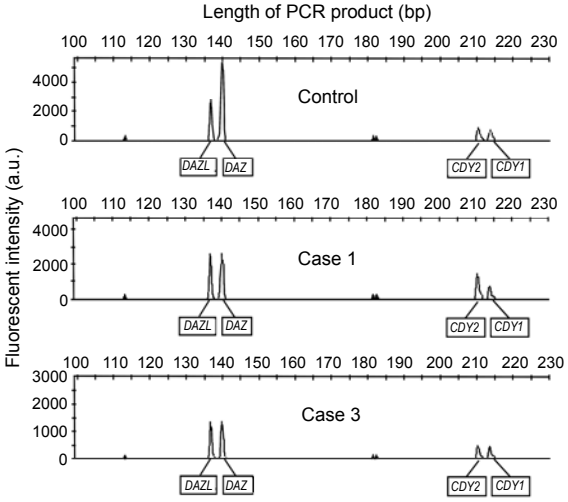

Fig. 3 Examples of electrophoretograms showing different gene dosages for the *DAZ*/*DAZL* and *CDY1*/*CDY2* genes

The peak areas reflect the dosage of PCR products of detected genes. Control: fertile man; Case 1: gr/gr deletion; Case 3: sY1206 deletion

Table 2 Distribution of deletion types of *DAZ* and *CDY1* in gr/gr and b2/b3 deletions

Group	Simple gr/gr deletion					Simple b2/b3 deletion				
	n	*DAZ1/2+ CDY1a*	*DAZ1/2+ CDY1b*	*DAZ3/4+ CDY1a*	*DAZ3/4+ CDY1b*	*n*	*DAZ1/2+ CDY1a*	*DAZ1/2+ CDY1b*	*DAZ3/4+ CDY1a*	*DAZ3/4+ CDY1b*
Fertile males	13	8	0	0	5	11	1	1	0	9
Azoospermia	3	1	1	0	1	1	0	0	0	1
Severe oligozoospermia	12	4	4	0	4	9	0	0	0	9
Infertile males	15	5	5	0	5	10	0	0	0	10

Table 3 Distribution of copy numbers of *DAZ* and *CDY1*

Group	*n*	6*DAZ*+3*CDY1*	4*DAZ*+2*CDY1*	4*DAZ*+1*CDY1*	2*DAZ*+2*CDY1*	2*DAZ*+1*CDY1*
Fertile males	153	1 (0.7%)	127 (83.0%)	1 (0.7%)	0	24 (15.7%)
Azoospermia	55	1 (1.8%)	49 (89.1%)	1 (1.8%)	0	4 (7.3%)
Severe oligozoospermia	169	8 (4.7%)*	138 (81.7%)	1 (0.6%)	1 (0.6%)	21 (12.4%)
Infertile males	224	9 (4.0%)*	187 (83.5%)	2 (0.9%)	1 (0.4%)	25 (11.2%)

* $P<0.05$, compared to the "fertile males" group

Table 4 Distribution of the subjects and partial *AZFc* deletions and duplications in Y chromosome haplogroups

Group	*n*	C	D	K-R*	O*	O1a	O2a	O3*	O3a*	O3a3*	O3a3b	O3a3c*	O3a3c1	Others
All														
Fertile males	115	12	2	26	4	6	16	3	17	5	3	8	8	5
Infertile males	215	19	6	52	7	10	22	5	30	11	6	16	19	12
Simple gr/gr deletion														
Fertile males	10	0	1	5	1	1	1	0	0	1	0	0	0	0
Infertile males	14	1	1	8	0	0	2	0	0	0	0	1	1	0
Simple b2/b3 deletion														
Fertile males	10	0	0	9	1	0	0	0	0	0	0	0	0	0
Infertile males	10	1	0	9	0	0	0	0	0	0	0	0	0	0
Partial duplication														
Fertile males	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Infertile males	9	2	0	2	1	1	0	1	1	0	0	0	1	0

The asterisk (*) indicates that the haplogroup may not be monophyletic

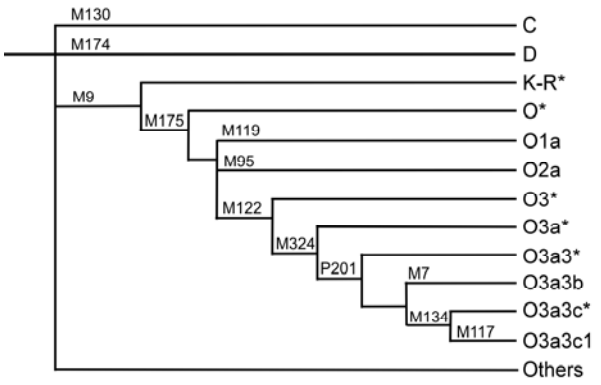

Fig. 4 Phylogenetic tree of Y chromosome haplogroups
The asterisk (*) indicates that the haplogroup may not be monophyletic

Eight cases out of 14 with simple gr/gr deletion and 9 cases out of 10 with b2/b3 deletion were found in 56 infertile members of haplogroup K-R*. Comparing to the control group, the 26 members of this group contributed 5 out of 10 gr/gr deletions and 9 out of 10 b2/3 deletions. Low frequencies of partial *AZFc* deletions were found in the other haplogroups. The distribution of partial *AZFc* duplications did not show significant differences between these haplogroups.

4 Discussion

Ethnic background is important for the association study between partial *AZFc* polymorphisms and male infertility. Different results have been observed in different ethnic populations (Navarro-Costa *et al*., 2010b). Yi ethnic minority, the 7th largest ethnic minority in China, has its own script and language which belongs to the Tibeto-Burman language group of the Chinese-Tibetan language family (Zhu *et al*., 2010). A total of 61% of Yi people reside in Yunnan Province (Zhu *et al*., 2010). In this study, we have focused on the Yi ethnic group by selecting cases as either non-obstructive azoospermia or severe oligozoospermia according to the WHO criteria. The controls are males who have given birth to at least one offspring without the help of assisted reproductive technology (confirmed by paternity test), but semen analysis was not performed. Thus, the control subjects are fertile but were not confirmed for normozoospermia. Our results show that the frequency of gr/gr deletion is 7.6% in cases, in contrast to 8.5% in controls, and the frequencies of the b2/b3 deletion are

6.3% and 8.5% respectively in these two groups (Table 1). The frequencies of gr/gr and b2/b3 deletions were similar to those in previous studies, but they did not differ significantly between the case and control groups, which agrees with most studies in East Asian population (Zhang *et al*., 2006) but conflicts with results published by Giachini *et al*. (2008) which have demonstrated the gr/gr deletion to be a significant risk for normal spermatogenesis in the Italian population using a similar methodology. This difference may be due to different genetic backgrounds, indicating a diverse sensitivity to the gr/gr deletion depending on genetic background. The haplogrouping results show that frequencies of gr/gr and b2/b3 deletions are low in the common East Asian group O* but significantly high in the group K-R*, which is consistent with previous reports (Table 4) (Lin *et al*., 2007; Zhang *et al*., 2007). These data suggest that the frequencies of partial *AZFc* deletion are significantly influenced by genetic background. Thus, haplotype match of cases and controls is of great importance in the association study on partial *AZFc* deletions and male infertility.

Besides the deletions occurring via homologous recombination between ampliconic sequences, deletions via non-allelic homologous recombination (NAHR) have been reported, and are due to the inherently unstable palindromic structure (Noordam *et al*., 2011). In this study, we found a new deletion type with the absence of sY1206 on a patient bearing severe oligozoospermia (Table 1). A similar deletion, named b3/b4 deletion, has been reported by Ferlin *et al*. (2005) as well as Choi *et al*. (2012). They speculated that the breakpoints of this type of deletion are located between sY1125 and sY1054 for the absence of sY1054 and presence of sY1125, and that this deletion removes the block Y1-g2-r3-r4-g3-Y2 including two copies of *CDY1* and *DAZ3/4*. The breakpoints of case 3 seem to locate between sY639 and sY1206. This deletion removes g2-r3-r4-g3, including *DAZ3/4* (Fig. 1f), which is consistent with the copy numbers of *DAZ* (two copies) and *CDY1* (two copies). Since the breakpoints locate at palindrome 1, deletion in case 3 and b3/b4 deletion seem to occur via NAHR mechanism (Navarro-Costa *et al*., 2010a).

We analyzed partial duplications by detecting copy numbers of *DAZ* and *CDY1*. We found more cases with partial duplications in infertile men (4.0%) than in controls (0.7%) (Table 3). Similar results have been reported by Lin *et al*. (2007) and replicated in the China-Han population (Zhang *et al*., 2007). When comparing the fertile subjects to azoospermic or severe oligozoospermic patients, a significantly higher occurrence of partial duplications is observed only in patients with oligozoospermia, similar with what occurs with gr/gr deletion (Stouffs *et al*., 2011). That the distribution of partial *AZFc* duplications does not show significant differences between these haplogroups reduces the influence of genetic background bias between cases and controls. Partial duplications increase the dosage of contained genes, such as *DAZ*, encoding RNA-binding proteins, and *CDY1*, encoding a histone acetyltransferase. These proteins seem to be involved in gene/protein synthesis regulation. Thus, increased dosage of these genes may disrupt the normal expression of targeted genes, which may affect normal spermatogenesis.

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Compliance with ethics guidelines

Jun-jie YE, Li MA, Li-juan YANG, Jin-huan WANG, Yue-li WANG, Hai GUO, Ning GONG, Wen-hui NIE, and Shu-hua ZHAO declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000(5). Informed consent was obtained from all patients for being included in the study.

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